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Title:

OLIGONUCLEOTIDES SPECIFIC FOR HEPATITIS B VIRUS

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OLIGONUCLEOTIDES SPECIFIC FOR HEPATITIS B VIRUS

FIELD OF THE INVENTION

5   This invention relates to hepatitis B virus.
More particularly, this invention relates to the
control of hepatitis B viral expression and
replication using oligonucleotides complementary
to particular regions of hepatitis B virus nucleic
acid.

10   BACKGROUND OF THE INVENTION

          Hepatitis B virus (HBV) is a compact,
enveloped DNA virus belonging to the Hepadnavirus
family. This virus is the major cause of chronic
15   liver disease and hepatocellular carcinoma world-
wide (Hoofnagle (1990) *N. Eng. J. Med.* **323**:337-339).
HBV is associated with acute and chronic hepatitis
and hepatocellular carcinoma, and may also be a
cofactor in the development of acquired immune
20   deficiency syndrome (Dienstag et al. in *Harrison's
Principles of Internal Medicine*, 13th Ed. (Isselbacher et
al., eds.) McGraw-Hill, NY, NY (1993) pp. 1458-
1483). At least 400 million people worldwide are
currently infected with HBV.
25

          There is no known treatment for acute
hepatitis. Antiviral therapy with interferon-α
has been used for chronic hepatitis, but has met
with only partial success, and there are
30   complications from such therapy. Short term
therapy with glucocorticoids may be beneficial in
conjunction with interferon therapy, but long term
treatment is limited by toxicological problems

(Dienstag et al. in *Harrison's Principles of Internal Medicine*, 13th Ed. (Isselbacher et al., eds.) McGraw-Hill, NY, NY (1993) pp. 1458-1483). Thus, emphasis has been placed on prevention through immunization.

New chemotherapeutic agents have been developed which are capable of modulating cellular and foreign gene expression (see, Zamecnik et al. (1978) *Proc. Natl. Acad. Sci. (USA)* **75**:280-284; Zamecnik et al. (1986) *Proc. Natl. Acad. Sci. (USA)* **83**:4143-4146; Goodchild et al. (1988) *Proc. Natl. Acad. Sci. (USA)* **85**:5507-5511). These agents, called antisense oligonucleotides, bind to target single-stranded nucleic acid molecules according to the Watson-Crick rule or to double-stranded nucleic acids by the Hoogsteen rule of base pairing, and in so doing, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of mRNA by RNase H, or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Improved oligonucleotides have more recently been developed that have greater efficacy in inhibiting such viruses, pathogens and selective gene expression. Some of these oligonucleotides having modifications in their internucleotide linkages have been shown to be more effective than their unmodified counterparts. For example, Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1988) **85**:7079-7083) teaches that oligonucleotide

phosphorothioates and certain oligonucleotide phosphoramidates are more effective at inhibiting HIV-1 than conventional phosphodiester-linked oligodeoxynucleotides. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1989) **86**:7790-7794) discloses the advantage of oligonucleotide phosphorothioates in inhibiting HIV-1 in early and chronically infected cells.

In addition, chimeric oligonucleotides having more than one type of internucleotide linkage within the oligonucleotide have been developed. Pederson et al. (U.S. Patent Nos. 5,149,797 and 5,220,007) discloses chimeric oligonucleotides having an oligonucleotide phosphodiester or oligonucleotide phosphorothioate core sequence flanked by nucleotide methylphosphonates or phosphoramidates. Furdon et al. (*Nucleic Acids Res.* (1989) **17**:9193-9204) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters in addition to either oligonucleotide phosphorothioate or methylphosphonate regions. Quartin et al. (*Nucleic Acids Res.* (1989) **17**:7523-7562) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters and oligonucleotide methylphosphonates. Inoue et al. (*FEBS Lett.* (1987) **215**:237-250) discloses hybrid oligonucleotides having regions of deoxyribonucleotides and 2'-O-methyl-ribonucleotides.

Antisense oligonucleotides have been designed which inhibit the expression and/or replication of

HBV. For example, antisense oligonucleotides directed against the cap site of HBV mRNA transcribed from the SPII promoter (Goodarzi et al. (1990) *J. Gen. Virol.* **71**:3021-3025; Yao et al.

5   (1994) *Nat. Med. J. China* **74**:125), against the translational initiation site of the S gene (Yao et al. (1994) *Nat. Med. J. China* **74**:125; Reinis et al. (1993) *Folia Biologica* (Praha) **39**:262-269; Goodarzi et al. (1990) *J. Gen. Virol.* **71**:3021-3025); against a

10   portion of the core-pol mRNA encoding the terminal protein region of the viral polymerase (WO 94/24864; Blum et al. (1991) *Lancet* **337**:1230), and against the HBV polyadenylation signal (Wu et al. (1992) *J. Biol. Chem.* **267**:12436-12439) have been

15   designed. In addition, phosphorothioate oligodeoxynucleotides prepared against the 5' region of the pre-S gene have been shown to inhibit duck HBV replication and gene expression *in vivo* (Offensperger et al. (1993) *EMBO J.* **12**:1257-

20   1262).

A need still remains for the development of oligonucleotides that are capable of inhibiting the replication and expression of HBV whose

25   administration are accompanied by a good prognosis and low or no cellular toxicity.

SUMMARY OF THE INVENTION

30   It has been discovered that specific oligonucleotides complementary to particular contiguous and noncontiguous portions of pregenomic and messenger RNA encoding the precore,

core, and polymerase proteins of HBV can inhibit
HBV replication, packaging, and expression. This
discovery has been exploited to provide synthetic
oligonucleotides complementary to various
5   contiguous and noncontiguous regions of HBV RNA.

As used herein, a "synthetic oligonucleotide"
includes chemically synthesized polymers of about
five and up to about 50, preferably from about 15
10   to about 30 ribonucleotide and/or
deoxyribonucleotide monomers connected together or
linked by at least one, and preferably more than
one, 5' to 3' internucleotide linkage.

15   For purposes of the invention, the term
"oligonucleotide sequence that is complementary to
RNA" is intended to mean an oligonucleotide that
binds to the nucleic acid sequence under
physiological conditions, e.g., by Watson-Crick
20   base pairing (interaction between oligonucleotide
and single-stranded nucleic acid) or by Hoogsteen
base pairing (interaction between oligonucleotide
and double-stranded nucleic acid) or by any other
means, including in the case of an oligonucleotide
25   binding to RNA, causing pseudoknot formation.
Binding by Watson-Crick or Hoogsteen base pairing
under physiological conditions is measured as a
practical matter by observing interference with
the function of the nucleic acid sequence.
30
In a first aspect, the invention provides
synthetic oligonucleotides complementary to a

portion of the HBV RNA and having a nucleotide
sequence selected from the group consisting of SEQ
ID NOS:1-31 and 42-48.

5   In another aspect, the invention provides
synthetic oligonucleotides complementary to at
least two noncontiguous regions of an HBV nucleic
acid. In preferred embodiments, the two
noncontiguous regions to which the
10   oligonucleotides of the invention are
complementary are in the epsilon region of the
precore gene. As used herein, the "epsilon
region" is meant to encompass the stem-loop and
flanking base sequences of the pregenomic RNA,
15   precpre mRNA, and core-pol mRNA, and includes
nucleotides (nt) 1827-1921. In some embodiments,
these oligonucleotides are about 20 to about 30
nucleotides in length. In some embodiments,
noncontiguous oligonucleotides of the invention
20   include a sequence selected from the group
consisting of SEQ ID NOS:32-41.

In some embodiments, the oligonucleotides of
the invention are modified. These modifications,
25   in some embodiments, include at least one
alkylphosphonate, phosphorothioate,
phosphorodithioate, alkylphosphonothioate,
phosphoramidate, carbamate, carbonate, phosphate
triester, acetamidate, or carboxymethyl ester
30   internucleotide linkage or a combination of such
linkages, as in a chimeric oligonucleotide. In
one preferred embodiment, an oligonucleotide of
the invention includes phosphorothioate
internucleotide linkages.

In some embodiments, the oligonucleotides of the invention also include at least one ribonucleotide, at least one deoxyribonucleotide, or a combination thereof, as in a hybrid

5   oligonucleotide. An oligonucleotide containing at least one 2'-O-methyl ribonucleotide is another embodiment of the invention.

In other aspects, the invention provides a

10   pharmaceutical composition comprising at least one contiguous or noncontiguous HBV-specific oligonucleotide of the invention as described above, and in some embodiments, this composition includes at least two different oligonucleotides

15   (i.e., having a different nucleotide sequence, length, and/or modification(s)). The pharmaceutical composition of some embodiments is a physical mixture of at least two, and preferably, many oligonucleotides with the same or

20   different sequences, modifications, and/or lengths. In some embodiments, this pharmaceutical formulation also includes a physiologically or pharmaceutically acceptable carrier.

25   Another aspect of the invention are kits for inhibiting HBV replication and/or infection in a cell. In preferred embodiments, the kits include at least one contiguous or noncontiguous oligonucleotide of the invention, or a combination

30   thereof. In other preferred embodiments, at least two synthetic oligonucleotides of the invention are in the kit.

In yet another aspect of the invention, a therapeutic amount of a pharmaceutical composition containing HBV-specific synthetic oligonucleotides is administered to the cell in a method of

5 inhibiting HBV replication. The HBV-specific oligonucleotides are the contiguous or noncontiguous oligonucleotides of the invention. In some preferred embodiments, the method includes administering at least one oligonucleotide, or at

10 least two oligonucleotides, having a sequence set forth in the Sequence Listing as SEQ ID NO:1-31, 32-41, or 42-48, or a combination thereof.

In another aspect, a method of treating HBV

15 infection is provided, comprising the step of administering to an infected animal, including a human, or cell, a therapeutic amount of a pharmaceutical composition containing at least one HBV-specific oligonucleotide, and in some

20 embodiments, at least two HBV-specific oligonucleotides. The HBV-specific oligonucleotides are contiguous or noncontiguous. In preferred embodiments, the oligonucleotides administered have a sequence set forth in the

25 Sequence Listing as SEQ ID NO:1-31, 32-41, or 42-48, or a combination thereof.

In all methods involving the administration of oligonucleotide(s) of the invention, at least

30 one, and preferably two or more identical or different oligonucleotides may be administered simultaneously or sequentially as a single

treatment episode in the form of separate
pharmaceutical compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a schematic representation showing contiguous oligonucleotides of the invention targeted to various functional regions of the HBV ayw sequence from nt 1786-2328;

FIG. 2 is a graphic representation showing sites on HBV RNA accessible to oligonucleotide hybridization detected by RNase H cleavage wherein the numbers represent specific HBV oligonucleotides of the invention;

FIG. 3 is a schematic representation showing the sequence and two-dimensional structure of the epsilon region;

FIG. 4A is a diagrammatic representation showing mode A of oligonucleotide binding to the base of DNA and RNA stems;

FIG. 4B is a diagrammatic representation showing mode B of oligonucleotide binding to the base of DNA and RNA stems;

FIG. 5A is a graphic representation showing the results of RNase H cleavage in the presence of noncontiguous oligonucleotides having 10 nucleotides complementary to the 5' side (site 1)

5   of the epsilon region and 10 nucleotides complementary to the 3' side of the epsilon region (10+10);

FIG. 5B is a graphic representation showing

10   the results of RNase H cleavage with 10+10 noncontiguous oligonucleotides (two cuts);

FIG. 6A is a graphic representation showing the results of RNase H cleavage in the presence of

15   noncontiguous oligonucleotides having 12 nucleotides complementary to the 5' side (site 1) of the epsilon region and 12 nucleotides complementary to the 3' side of the epsilon region (12+12);

20

FIG. 6B is a graphic representation showing the results of RNase H cleavage with 12+12 noncontiguous oligonucleotides (two cuts);

25   FIG. 7 is a graphic representation showing the inhibitory effect of different concentrations of HBVpol-2 on the translation of HBVpol RNA, wherein peak areas are arbitrary units;

30

FIG. 8 is a graphic representation showing the inhibitory effect of different concentrations of HBVpol-2 and related mismatched oligonucleotides, HBVpol-A, HBVpol-B, HBVpol-C, and HBVpol-D, on the translation of HBVpol RNA, wherein the dark bars represent translation of control RNA, and the hatched bars represent translation of HBVpol test RNA, wherein peak areas are arbitrary units;

FIG. 9 is a graphic representation showing the inhibitory effect of contiguous oligonucleotides of the invention (HBVpol-1, HBVpol-2, and HBVpol-3) on luciferase expression;

FIG. 10 is a schematic representation of the HBV-luciferase fusion targets used for luciferase assays;

FIG. 11 is a graphic representation showing the results of a Southern hybridization assay demonstrating inhibition of the formation of replicative intermediate (RI) HBV DNA in HepG2.2.15 cells in the presence of different concentrations of HBV6;

FIG. 12 is a graphic representation showing the results of a Southern hybridization assay demonstrating inhibition of the formation of replicative intermediate (RI) HBV DNA in HepG2.2.15 cells in the presence of different concentrations of HBV67; and

FIG. 13 is a diagrammatic representation of the map of pHBVpol.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references cited herein are hereby incorporated by reference.

HBV is a compact, enveloped DNA virus belonging to the Hepadnavirus family. It has a circular, partially single-stranded, partially double-stranded 3.2 kb genome which includes four overlapping genes: (1) the pre-S and S genes, which encode the various envelope or surface antigens (HBsAg); (2) the preC and C gene, which encodes the antigens HBcAg and HBeAg; (3) the P gene, which encodes the viral polymerase; and (4) the X gene, which encodes HBx, the transactivating protein. Full-length clones of many hepadnaviruses have been obtained and their nucleotide sequences obtained. (see, e.g., Raney et al. in *Molecular Biology of the Hepatitis B Virus* (McLachlan, ed.) CRC Press, Boston, MA, (1991) pp. 1-38). Replication occurs in hepatocytes and involves converting the single stranded-region of the HBV genome to double-stranded circular DNA, generating the covalently closed circular (CCC) DNA. Transcription of this DNA by the host RNA polymerase generates an RNA template of plus stranded polarity, the pregenomic RNA, which serves as a template for the translation of viral proteins, and is also encapsidated into virus cores. In the virus cores, the RNA serves as a

template for reverse transcription, generating a
DNA minus strand. The viral polymerase then
produces a DNA plus strand using an oligomer of
viral RNA as a primer. The newly synthesized
5   double-stranded DNA in the viral core is assembled
with the viral envelope proteins, generating a
newly infectious viral particle.

Antisense oligonucleotide technology provides
10   a novel approach to the inhibition of HBV
expression, and hence, to the treatment or
prevention of acute and chronic hepatitis and
hepatocellular carcinoma (see generally, Agrawal
(1992) *Trends Biotech.* **10**:152; and Crooke (*Proc. Am. Ass.*
15   *Cancer Res. Ann. Meeting* (1995) **36**:655). By binding to
the complementary nucleic acid sequence, antisense
oligonucleotides are able to inhibit splicing and
translation of RNA, and replication of genomic
RNA. In this way, antisense oligonucleotides are
20   able to inhibit protein expression.

Synthetic "contiguous" oligonucleotides of
the invention, or oligonucleotides targeted to
contiguous regions of HBV precore mRNA, core/pol
25   mRNA, and pregenomic RNA (FIG. 1) are
complementary to sequences encoding the precore
region, to sequences spanning the precore and core
regions, to sequences including the polymerase
translation initiation region, and to regions
30   within the epsilon region. Representative
contiguous oligonucleotides of the invention are
set forth below in Table 1.

TABLE 1

OLIGO	Sequence (5' → 3')	Position	Chemistry	SEQ ID NO:
HBV49[a]	GGTGCGCAGACCAATTTATG	1790-1809	DNA PS	1
HBV45[a]	CATGGTGCTGGTGCGCAGA	1799-1818	DNA PS	2
HBV44[a]	GAAAAAGTTGCATGGTGCTG	1809-1828	DNA PS	3
HBV48[a]	GAGGTGAAAAAGTTGCATGG	1814-1833	DNA PS	4
HBV47[a]	AGGCAGAGGTGAAAAAGTTG	1819-1838	DNA PS	5
HBV72[a]	AGGCAGAGGTGA	1827-1838	DNA PS	6
HBV43[a]	AGAGATGATTAGGCAGAGGT	1829-1848	DNA PS	7
HBV43M[a]	AGAGAUGAUUAGGCAGAGGT	1829-1848	2'-OME PS/DNA PS	7
HBV88[b]	GACATGAACAAGAGATGATTAGGCAGAGGT	1829-1858	DNA PS	8
HBV88M[b]	GACAUGAACAAGAGAUGAUUAGGCAGAGGT	1829-1858	2'-OME PS/DNA PS	8
HBV46[c]	GACATGTACAAGAGATGATT	1839-1858	DNA PS	9
HBV46Y[b]	GACATGAACAAGAGATGATT	1839-1858	DNA PS	9
HBV46MY[b]	GACAUGAACAAGAGAUGAUU	1839-1858	2'-OMe PS	9
HBV1[b]	GTAGGACATGAACAAGAGAT	1843-1862	DNA PS	10
HBV2[b]	TTGGAGGCTTGAACAGTAGG	1858-1877	DNA PS	11
HBV5[a]	CACAGCTTGGAGGCTTGAAC	1864-1883	DNA PS	12
HBV3[a]	AGCCACCCAAGGCACAGCTT	1876-1895	DNA PS	13
HBV4[b]	TCGATGTCCATGCCCCAAAG	1894-1913	DNA PS	14
HBV92[b]	TAAGGGTCGATGTCCATGCC	1900-1919	DNA PS	15
HBV92M[b]	TAAGGGTCGAUGUCCAUGCC	1900-1919	2'-OMe PS/DNA PS	15
HBV92M2[b]	TAAGGGUCGAUGUCCATGCC	1900-1919	2'-OMe PS/DNA PS	15
HBV101[b]	TTATAAGGGTCGATGTCCAT	1903-1922	DNA PS	16
HBV101M[b]	TTATAAGGGTCGAUGUCCAU	1903-1922	2'-OMe PS/DNA PS	16
HBV94[b]	AAATTCTTTATAAGGGTCGATGTCCAT	1903-1929	DNA PS	17
HBV71[b]	TATAAGGGTCGA	1910-1921	DNA PS	18
HBV93[b]	AAATTCTTTATAAGGGTCGA	1910-1929	DNA PS	19
HBV93M[b]	AAATTCTTTATAAGGGUCGA	1910-1929	2'-OMe PS/DNA PS	19
HBV61[b]	GTATCTAGAAGATCTCGTAC	1981-2000	DNA PS	20
HBV60[c]	GCGGTGTCTAGAAGATCTCG	1984-2003	DNA PS	21
HBV60Y[b]	GCGGTATCTAGAAGATCTCG	1984-2003	DNA PS	21
HBV57[c]	GAGGCGGTGTCTAGGAGATC	1987-2006	DNA PS	22
HBV57Y[b]	GAGGCGGTATCTAGAAGATC	1987-2006	DNA PS	22
HBV42[c]	GAGCTGAGGCGGTGTCTAGG	1992-2011	DNA PS	23
HBV42Y[b]	GAGCTGAGGCGGTATCTAGA	1992-2011	DNA PS	23
HBV54[b]	ATACAGAGCTGAGGCGGTAT	1997-2016	DNA PS	24
HBV55[b]	TCCCGATACAGAGCTGAGGC	2002-2021	DNA PS	25
HBV56[b]	AGGCTTCCCGATACAGAGCT	2007-2026	DNA PS	26
HBV53[b]	ACAATGCTCAGGAGACTCTA	2027-2046	DNA PS	27
HBV41[c]	GCAGTATGGTGAGGTGAGCA	2044-2063	DNA PS	28
HBV41Y[b]	GCAGTATGGTGAGGTGAACA	2044-2063	DNA PS	28
HBV51[a]	GAGTGCAGTATGGTGAGGTG	2048-2067	DNA PS	29
HBV50[a]	TGCCTGAGTGCAGTATGGTG	2053-2072	DNA PS	30
HBV52[b]	TTGCTTGCCTGAGTGCAGTA	2058-2077	DNA PS	31
HBVpol-1	GGCATTTGGTGGTCTATAAG	2294-2314	DNA PS	42
HBVpol-2	GATAGGGGCATTTGGTGGTC	2300-2319	DNA PS	43
HBVpol-3	TGTTGATAGGATAGGGGCAT	2309-2328	DNA PS	44
HBV6	ACCCAAGGCACAGCTTGGAG	1872-1891	DNA PS	45
HBVpol-A[b]	GAcAGGGGCATTTGGTGGTC	2300-2319	DNA PS	46
HBVpol-B[b]	GATAGGGGCcTTTGGTGGTC	2300-2319	DNA PS	47
HBVpol-C[b]	GATAGGGGCATTTGGTGcTC	2300-2319	DNA PS	48
HBVpol-D[b]	GAcAGGGGCcTTTGGTGcTC	2300-2319	DNA PS	49
HBV69	TAAGGGTCGA	1910-1919	DNA PS	53
HBV73	AGGCAGAGGT	1829-1838	DNA PS	54

[a] - target strain = ayw and adw
[b] - target strain = ayw
[c] - target strain = adw
underscoring = 2'-OMe RNA PS
N = PS DNA
lower case letters indicate mismatched nucleotides

Sequence positions listed in Table 1 represent the
standard orientation as shown by Raney et al. in
Molecular Biology of the Hepatitis B Virus (McLachlan,
ed.(1991): CRC Press, Boca Raton, FL. Ch 1, pp 2-
5   37). Synthetic "noncontiguous" oligonucleotides
of the invention target noncontiguous portions of
the epsilon region, and within this region, bind
across the base of the stem loop and from the base
to within the stem.

10

Representative noncontiguous oligonucleotides
of the invention are set forth below in Table 2.

TABLE 2

Oligo	Sequence (5'→3')	Site 1 (5')	Site 2 (3')	SEQ ID NO:
HBV-19[b]	TAAGGGTCGAAGAGATGATT	1839-1848	1910-1919	32
HBV-64[b]	AGAGATGATTTAAGGGTCGA	1839-1848	1910-1919	33
HBV-64M1[b]	AGAGATGATTUAAGGGUCGA	1839-1848	1910-1919	33
HBV-64M2[b]	AGAGATGATTTAAGGGUCGA	1839-1848	1910-1919	33
HBV-68[b]	TAAGGGTCGAAGGCAGAGGT	1829-1838	1910-1919	34
HBV-66[b]	AGGCAGAGGTTAAGGGTCGA	1829-1838	1910-1919	35
HBV-79[b]	TATAAGGGTCGAAGGCAGAGGTGA	1827-1838	1910-1921	36
HBV-67[b]	AGGCAGAGGTGATATAAGGGTCGA	1827-1838	1910-1921	37
HBV-67M1[b]	AGGCAGAGGTGAUAUAAGGGUCGA	1827-1838	1910-1921	37
HBV-67M2[b]	AGGCAGAGGUGAUATAAGGGTCGA	1827-1838	1910-1921	37
HBV-87[b]	AGAGATGATTAGGCAGAGGTTAAGGGTCGA	1829-1848	1910-1921	38
HBV-87M[b]	AGAGAUGAUUAGGCAGAGGTTAAGGGTCGA	1829-1848	1910-1921	38
HBV-89[b]	GACATGAACAAGAGATGATTTAAGGGTCGA	1839-1858	1910-1921	39
HBV-89M[b]	GACAUGAACAAGAGAUGAUUTAAGGGTCGA	1839-1858	1910-1921	39
HBV-90[b]	AGAGATGATTTAAGGGTCGATGTCCATGCC	1839-1848	1900-1919	40
HBV-90M[b]	AGAGAUGAUUTAAGGGTCGAUGUCCAUGCC	1839-1848	1900-1919	40
HBV-91[b]	AGGCAGAGGTTAAGGGTCGATGTCCATGCC	1829-1838	1900-1919	41
HBV-91M[b]	AGGCAGAGGTTAAGGGTCGAUGUCCAUGCC	1829-1838	1900-1919	41

[a] target strain = ayw and adw
[b] target strain = ayw
[c] target strain = adw
underscoring = 2'-OMe RNA PS
N = PS DNA

Synthetic oligonucleotides of the invention specific for HBV nucleic acid are composed of deoxyribonucleotides, ribonucleotides, 2'-O-methyl-ribonucleotides, or any combination

5   thereof, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked. These oligonucleotides are at least 6 nucleotides in length, but are preferably 12 to 50 nucleotides long, with 20 to 30mers being the most

10   common.

These oligonucleotides can be prepared by art recognized methods. For example, nucleotides can be covalently linked using art recognized

15   techniques such as phosphoramidite, H-phosphonate chemistry, or methylphosphoramidite chemistry (see, e.g., Goodchild (1990) *Bioconjugate Chem.* **2**:165-187; Uhlmann et al. (1990) *Chem. Rev.* **90**:543-584; Caruthers et al. (1987) *Meth. Enzymol.* **154**:287-313;

20   U.S. Patent 5,149,798) which can be carried out manually or by an automated synthesizer and then processed (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* **10**:152-158).

25   The oligonucleotides of the invention may also be modified in a number of ways without compromising their ability to hybridize to HBV nucleic acid. For example, the oligonucleotides may contain other than phosphodiester

30   internucleotide linkages between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups, such

as a phosphorothioate. Oligonucleotides with phosphorothioate linkages can be prepared using methods well known in the field such as phosphoramidite (*see, e.g.*, Agrawal et al. (1988)

5 *Proc. Natl. Acad. Sci.* (USA) **85**:7079-7083) or H-phosphonate (*see, e.g.*, Froehler (1986) *Tetrahedron Lett.* **27**:5575-5578) chemistry. The synthetic methods described in Bergot et al. (*J. Chromatog.* (1992) **559**:35-42) can also be used. Examples of other

10 chemical groups include alkylphosphonates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, 2'-O-methyls, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with

15 modified internucleotide linkages can be prepared according to known methods (see, e.g., Goodchild (1990) *Bioconjugate Chem.* **2**:165-187; Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1988) **85**:7079-7083); Uhlmann et al. (*Chem. Rev.* (1990) **90**:534-583; and

20 Agrawal et al. (*Trends Biotechnol.* (1992) **10**:152-158)).

Other modifications include those which are internal or are at the end(s) of the oligonucleotide molecule and include additions to

25 the molecule at the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the two amino groups, and terminal ribose, deoxyribose and phosphate modifications which

30 cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a

modified base and/or sugar such as arabinose
instead of ribose, or a 3', 5'-substituted
oligonucleotide having a sugar which, at one or
both its 3' and 5' positions is attached to a
5   chemical group other than a hydroxyl or phosphate
group (at its 3' or 5' position). Other modified
oligonucleotides are capped with a nuclease
resistance-conferring bulky substituent at their
3' and/or 5' end(s), or have a substitution in one
10   or both nonbridging oxygens per nucleotide. Such
modifications can be at some or all of the
internucleoside linkages, as well as at either or
both ends of the oligonucleotide and/or in the
interior of the molecule (reviewed in Agrawal et
15   al. (1992) *Trends Biotechnol.* **10**:152-158).

To determine whether an oligonucleotide of
the invention is capable of successfully
hybridizing to its target, an RNase H assay was
20   performed (Frank et al. (1993) *Proc. Int. Conf. Nucleic
Acid Med. Applns.* **1**:4.14(abstract)). This assay is
useful when a region of at least four contiguous
nucleotides of the oligonucleotide is DNA and the
target is RNA. Hybridization of the DNA portion
25   of the oligonucleotide to the RNA target is
identified by cleavage at that site by RNase H.

In vitro transcribed HBV RNA (adw strain) was
probed for sites accessible to oligonucleotide
30   hybridization using a randomized library of 20
base oligodeoxynucleotides (approximately 4^{20}
sequences). Hybridization to the RNA was detected
by RNase H cleavage of the end-labelled
transcript. Three regions were identified by this

assay. One region was in the 5' untranslated
region, between 113 and 70 bases upstream from the
core initiator, and two regions were in the coding
regionfor core, between 78 and 174 bases
5   downstream from the core initiator. Contiguous
oligodeoxynucleotide phosphorothioates were
prepared against these regions and their ability
to activate RNase H cleavage of the transcript
measured. The results shown in FIG. 2 demonstrate
10 single peaks of activity in each region,
corresponding to nt 1809-1828 (HBV44, SEQ ID NO:3)
in the 5' untranslated region, and nt 1987-2006
(HBV57, SEQ ID NO:22) and nt 2044-2063 (HBV41,
SEQ ID NO:28) in the coding region for core.
15

    Noncontiguous oligonucleotides targeted to
the HBV epsilon region have also been prepared and
tested. The epsilon region is characterized by an
RNA stem-loop structure consisting primarily of
20 double-stranded RNA with a single-stranded bulge
and loop of 6 bases each (FIG. 3). Two modes of
hybridization of these oligonucleotides have been
discovered and are shown in FIG. 4A and 4B. Mode
B in FIG. 4B appears to be preferred as
25 demonstrated by the cleavage of the RNA by
ribonuclease H on both sides of the stem. The
ability of RNase H to cleave an RNA in this manner
inflicts greater damage on the RNA than normal
antisense oligodeoxynucleotides, while allowing
30 the targeting of a biologically important region
that is otherwise difficult to target due to its
double-stranded nature.

Semirandom oligonucleotides consist of a
defined sequence of 2'-O-methyl ribonucleotides
and an undefined tail synthesized as a mixture of
all four deoxyribonucleosides at each position.

5   The 2'-O-methyl portion serves as a sequence-
specific anchor, unable to activate RNase H. The
random DNA sequence can be on the 3' or 5' side of
the defined 2'-O-methyl sequence allowing for
hybridization to nearby sequences.   Hybridization

10   of the DNA portion to RNA is identified by
cleavage at that site by RNase H.

The RNase H cleavage assay was used to test
the ability of oligonucleotides to bind across the

15   base of the well characterized RNA hairpin
structure found in the epsilon region of HBV
pregenomic and messenger RNA (FIG. 3).   It was
expected that a semirandom oligonucleotide
targeted to the sequence 5' of the epsilon stem

20   might target the sequence 3' of the stem when the
random DNA sequence was on the 5' end of the
oligonucleotide, as shown in FIG. 4A, mode A.

Surprisingly, the experiments showed that

25   cleavage was seen on the 3' side of the epsilon
region only when the random portion of the
oligonucleotide was on the 3' end, hybridizing as
shown in FIG. 4B, mode B.   The converse was also
true.   When the 2'-O-methyl portion was targeted

30   to the sequence on the 3' side of epsilon,
cleavage was seen on the 5' side only with the

random DNA sequence on the 5' side of the
semirandomer.

   Based on this information, several
5 oligodeoxynucleotide phosphorothioates were
   prepared to test the hypothesis that hybridization
   by mode B is preferred when spanning the base of
   an RNA stem. 20mer and 24mer noncontiguous
   oligonucleotides (Table 2) were prepared as well
10 as the 10mers and 12mers corresponding to the
   "arms" of the noncontiguous sequences.

   The ability of these oligonucleotides to
   activate cleavage of internally ^{32}P-labelled HBV
15 precore-core RNA was tested in the presence of
   RNase H. FIG. 5A shows total RNA cleavage with
   10+10 noncontiguous oligodeoxynucleotide
   phosphorothioates (ten nucleotides at the 5' end
   targeting 3' of the RNA stem and the next ten
20 nucleotides at the 3' end targeting 5' of the RNA
   stem). Both noncontiguous oligonucleotides, HBV66
   (SEQ ID NO:35) and HBV68 (SEQ ID NO:34), activate
   RNase H cleavage of the transcript more
   effectively than the mixture of 10mer arms, HBV69
25 (SEQ ID NO:53) and HBV73 (SEQ ID NO:54). For the
   12+12 oligonucleotides (FIG. 6A and 6B), RNA
   cleavage activated by the mixture of 12mer arms,
   HBV71 (SEQ ID NO:18) and HBV72 (SEQ ID NO:6), is
   equal to the RNA cleavage in the presence of the
30 noncontiguous oligodeoxynucleotide
   phosphorothioates (HBV79, SEQ ID NO:36 and HBV67
   (SEQ ID NO:37) (FIG. 6A).

If a single oligodeoxynucleotide were able to
bind across the base of epsilon to sequences on
either side of the stem, RNase H might cleave both
sites and effectively cut out the epsilon stem-
5   loop from the RNA. The results of double cleavage
of HBV precore-core RNA labelled internally with
[α-^{32}P] dCTP are shown in FIG. 5B and 6B. The
efficiency of production of the twice cleaved
product by RNase H in the presence of HBV66 (the
10   noncontiguous 10+10 oligonucleotide hybridizing by
mode B) was greater after 10 minutes than in the
presence of HBV68 (the 10+10 noncontiguous
oligonucleotide hybridizing by mode A) (FIG. 5B).
The mixture of the individual 10mer arms was
15   unable to activate cleavage on both sides of the
same stem-loop (HBV69 + HBV73) (FIG. 5B). The
12+12 noncontiguous phosphorothioates show the
same ability to bind across the base of the RNA
stem. As shown in FIG. 6B, HBV79 (SEQ ID NO:36)
20   and HBV67 (SEQ ID NO:37) efficiently activate
RNase H cleavage on both sides of the stem after
only 1.5 minutes, with hybridization by mode B
(HBV67) showing slightly more cleavage than mode A
(HBV79). Double cleavage of the transcript in the
25   presence of the mixture of 12mer arms (HBV71 +
HBV72) was much slower (FIG. 6B).

When oligonucleotides hybridizing via mode B
were lengthened to allow strand invasion of the
30   RNA stem, disruption of the stem-loop structure
occurred. Oligonucleotides HBV89 (SEQ ID NO:39),
HBV90 (SEQ ID NO:40), and HBV91 (SEQ ID NO:41)
bind across the base of epsilon via mode B and
strand invade on either the 5' side (HBV89) or the

3' side (HBV90 and HBV91) of the RNA stem. HBV89M
is an extension of HBV64M with ten 2'-O-methyl RNA
residues invading the RNA stem on the 5'-side.
Addition of these strand invading nucleotides
5   increased the cleavage efficiency from 23% to 32%
at 100 nM oligonucleotide. HBV90 and HBV91 PS
were also able to strand invade as evidenced by
RNase H cleavage within the stem near the core
initiator.
10

        The oligonucleotides of the invention can be
assayed for antisense inhibitory activity with a
number of different assays. For example, an *in vitro*
translation assay can be used to test antisense
15   activity in which an antisense oligonucleotide can
inhibit synthesis of a protein product encoded by
the targeted mRNA. In such an assay,
oligonucleotides targeted to the polymerase gene
were tested against both target and an unrelated
20   control RNA in the wheat germ translation system.
In this assay, the contiguous oligonucleotide
HBVpol-2 (SEQ ID NO:43) at 400 nM showed good
specific activity causing between 70% and 100%
translation inhibition. Results are represented
25   graphically in FIG. 7.

        This assay was also used to compare the
activity of mismatched oligonucleotides to the
activity of the parent oligonucleotide. Four
30   such oligonucleotides, all derivatives of HBVpol-2
(HBVpol-A, SEQ ID NO:46; HBVpol-B, SEQ ID NO:47;
HBVpol-C, SEQ ID NO:48, HBVpol-D, SEQ ID NO:49)
(Table 1) were synthesized and tested in the
assay. The results are shown in FIG. 8. Those

oligonucleotides with a single mismatch (SEQ ID
NO:46-48) showed varying degrees of reduction in
activity when compared to HBVpol-2 (SEQ ID NO:43).
Three mismatches in the oligonucleotide (see SEQ

5   ID NO:49) abrogated antisense activity.

     Oligonucleotides targeted to the polymerase
translation initiation region were also tested in
mammalian cells using a firefly luciferase

10   reporter gene assay. The 35 nucleotide region
spanning the translation start site of the HBV ayw
polymerase gene from nt 2294-2328 was cloned 5'
to, and in frame with, the entire open reading
frame of the firefly luciferase gene in the

15   plasmid pGLori, to produce the plasmid pGLpol
(FIG. 10). Transcription of this pol-luciferase
gene fusion was placed under the control of the
cytomegalovirus early gene promoter. Expression
of the pol-luciferase fusion in mammalian cells

20   was quantified in a luminometer by addition of
luciferin substrate and ATP cofactor to cell
lysates. In all cellular antisense assays, a
random sequence 20mer phosphorothioate
oligonucleotide (random 20mer PS) was used as a

25   negative control. In addition, a 20mer
phosphorothioate antisense oligonucleotide
targeting the first 20 nucleotides of the coding
region of the firefly luciferase gene was used as
a positive control (Luc+1 - +20; SEQ ID NO:50).

30   This target is retained in both pol fusion and
control luciferase constructs. The reduction in
luciferase levels in cells treated with antisense
oligonucleotides compared to luciferase levels in
cells treated with a negative control random

oligonucleotide is a measure of the sequence specific activity of the antisense oligonucleotides.

5   Oligonucleotides of the invention were tested against the HBV subtype ayw polymerase gene-luciferase fusion construct in stably transfected HepG2 cells. The results are shown in FIG. 9. HBVpol-1 (SEQ ID NO:42) and HBVpol-2 (SEQ

10   ID NO:43) had sequence-specific antisense activity. None of these PS oligonucleotides, with the exception of the positive control Luc +1 - +20 oligonucleotide, exhibited antisense activity in HepG2 cells stably transfected with the parent

15   pGLori sequence.

       In addition to the HBVpol-luciferase fusion construct, three different HBV-luciferase fusion constructs were generated incorporating the region

20   around the HBV subtype ayw epsilon region (FIG. 10). The pGLE construct consists of 71 nucleotides representing the epsilon stem loop region (nt 1843-1913), inserted between the cytomegalovirus immediate early gene promoter and

25   luciferase reporter gene in the plasmid pGLori. Translation in this construct should initiate at the HBV core gene initiation site (nt 1903).

       The constructs pGLE2 and pGLE3 (FIG. 10)

30   consist of 130 nucleotides representing the precore translation start site and epsilon stem loop region (nt 1813-1943) inserted between the cytomegalovirus immediate early gene promoter and luciferase reporter gene in the plasmid pGLori.

In these two constructs the translation start site
of the luciferase gene was removed. In addition,
the HBV core gene translation start site was
mutated in pGLE3 (nt 1904: T → C). A

5   complementary mutation was introduced at nt 1854
(A → G) to maintain the base pairing in the
epsilon stem. In pGLE2 translation can be
initiated from the precore or core translation
start site. In pGLE3 translation can only be

10   initiated at the precore translation start site.

     The activity of antisense oligonucleotides
was also studied in a viral assay in HepG2.2.15
cells, which have been stably transfected with

15   plasmids carrying whole HBV genomes (Sells et al.
(1987) *Proc. Nat. Acad. Sci.* **84**:1005-1009; Sureau et al.
(1986) Cell **47**:37-47). While a number of assays
for HBV inhibitors based on the HepG2 2.2.15 cell
line have been reported (Jansen et al. (1993)

20   *Antimicrob. Agent. Chemother.* **37**:441-447; Korba et al.
(1992) *Antiviral Res.* **19**:55-70), these involve the
detection of HBV DNA by means of dot blot or PCR,
tests which do not provide data concerning the
precise source of the measured DNA. A more

25   definitive test is Southern hybridization, which
provides data concerning the character of the
detected DNA in addition to quantitation. This
assay has been described previously for the
screening of anti-HBV compounds on HepG2.2.15

30   cells (Doong et al. (1991) *Proc. Nat. Acad. Sci.* (USA)
88:8495-8499). In view of the many potential
sources of HBV DNA from transfected cells, this
assay allows for a more meaningful interpretation

of results than the other methods mentioned. When HBV6 (SEQ. ID NO:32) was titrated, significant inhibition was found (FIG. 11). Inhibition was also found to be mediated by the stem-loop

5   bridging oligonucleotide, HBV67 (SEQ. ID NO:37) (FIG. 12).

In addition to Southern hybridizations, kinetic PCR was performed to assay the

10 supernatants from the HepG2.2.15 cells. This procedure was carried out as described by Higuchi et al. (*Biotechnol.* (1993) **11**:1026-1030). All PCRs were carried out with two sets of external controls which consisted of a dilution series of a

15 known concentration of plasmid DNA that contained the HBV core gene amplified with the same primer set. These controls generated a standard curve that was used to calculate the copy number of HBV genomes in the supernatants from cells exposed to

20 the various dilutions of compound. From these data, IC_{50} values were calculated for each compound and are shown below in Table 3.

TABLE 3

25

SEQ ID NO:	Oligo	IC_{50} (μM)
3	HBV44	0.7
4	HBV48	0.7
18	HBV4	1.2
42	HBVpol-2	3.7
	Randomer	4.5

The results of this experiment demonstrate that the HBV-specific oligonucleotides of the invention have inhibitory activity.

5   The synthetic antisense oligonucleotides of the invention may be in the form of a therapeutic composition or formulation useful in inhibiting HBV replication in a cell, and in treating hepatitis B infections and associated conditions

10   in an animal, such as acute and chronic hepatitis and hepatocellular carcinoma. They may be used as part of a pharmaceutical composition when combined with a physiologically and/or pharmaceutically acceptable carrier. The characteristics of the

15   carrier will depend on the route of administration. Such a composition may contain, in addition to the synthetic oligonucleotide and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials

20   well known in the art. The pharmaceutical composition of the invention may also contain other active factors and/or agents which enhance inhibition of HBV expression. For example, combinations of synthetic oligonucleotides, each

25   of which is directed to different regions of the HBV nucleic acid, may be used in the pharmaceutical compositions of the invention. The pharmaceutical composition of the invention may further contain other chemotherapeutic drugs for

30   the treatment of hepatocellular carcinoma. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with the synthetic oligonucleotide of the invention, or to minimize

side-effects caused by the synthetic
oligonucleotide of the invention. Conversely, the
synthetic oligonucleotide of the invention may be
included in formulations of a particular anti-HBV
5   or anti-cancer factor and/or agent to minimize
side effects of the anti-HBV factor and/or agent.

The pharmaceutical composition of the
invention may be in the form of a liposome in
10   which the synthetic oligonucleotides of the
invention are combined, in addition to other
pharmaceutically acceptable carriers, with
amphipathic agents such as lipids which exist in
aggregated form as micelles, insoluble monolayers,
15   liquid crystals, or lamellar layers which are in
aqueous solution. Suitable lipids for liposomal
formulation include, without limitation,
monoglycerides, diglycerides, sulfatides,
lysolecithin, phospholipids, saponin, bile acids,
20   and the like. Preparation of such liposomal
formulations is within the level of skill in the
art, as disclosed, for example, in U.S. Patent No.
4,235,871; U.S. Patent No. 4,501,728; U.S. Patent
No. 4,837,028; and U.S. Patent No. 4,737,323. The
25   pharmaceutical composition of the invention may
further include other lipid carriers, such as
Lipofectamine, or cyclodextrins (Zhao et al.
(1995) *Antisense Res. Dev.* (in press)) and the like
which enhance delivery of oligonucleotides into
30   cells, or such as slow release polymers.

As used herein, the term "therapeutically
effective amount" means the total amount of each
active component of the pharmaceutical composition

or method that is sufficient to show a meaningful
patient benefit, i.e., a reduction in pain
associated with acute or chronic hepatitis or the
remission of hepatocellular carcinoma. When
5   applied to an individual active ingredient,
administered alone, the term refers to that
ingredient alone. When applied to a combination,
the term refers to combined amounts of the active
ingredients that result in the therapeutic effect,
10   whether administered in combination, serially or
simultaneously.

      In practicing the method of treatment or use
of the present invention, a therapeutically
15   effective amount of one or more of the synthetic
oligonucleotides of the invention is administered
to a subject afflicted with an HBV-associated
disease. The synthetic oligonucleotide of the
invention may be administered in accordance with
20   the method of the invention either alone or in
combination with other known therapies for the
HBV-associated disease. When co-administered with
one or more other therapies, the synthetic
oligonucleotide of the invention may be
25   administered either simultaneously with the other
treatment(s), or sequentially. If administered
sequentially, the attending physician will decide
on the appropriate sequence of administering the
synthetic oligonucleotide of the invention in
30   combination with the other therapy.

      It may be desirable at times to use a mixture
of different oligonucleotides targeting different
conserved sites within a given viral genome. Such

a mixture of oligonucleotides may be in the form
of a therapeutic composition comprising at least
one, and preferably two or more oligonucleotides
in a single therapeutic composition (i.e., a
5   composition comprising a physical mixture of at
least two oligonucleotides). These
oligonucleotides may have the same or different
sequences. At least one, preferably two or more
oligonucleotides may be administered
10 simultaneously or sequentially as a single
treatment episode in the form of separate
pharmaceutical compositions.

     Administration of the synthetic
15 oligonucleotide of the invention used in the
pharmaceutical composition or to practice the
method of treating an animal can be carried out in
a variety of conventional ways, such as oral
ingestion, inhalation, or cutaneous, subcutaneous,
20 intramuscular, or intravenous injection.

     When a therapeutically effective amount of
synthetic oligonucleotide of the invention is
administered orally, the synthetic oligonucleotide
25 will be in the form of a tablet, capsule, powder,
solution or elixir. When administered in tablet
form, the pharmaceutical composition of the
invention may additionally contain a solid carrier
such as a gelatin or an adjuvant. The tablet,
30 capsule, and powder contain from about 5 to 95%
synthetic oligonucleotide and preferably from
about 25 to 90% synthetic oligonucleotide. When
administered in liquid form, a liquid carrier such
as water, petroleum, oils of animal or plant

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origin such as peanut oil, mineral oil, soybean
oil, sesame oil, or synthetic oils may be added.
The liquid form of the pharmaceutical composition
may further contain physiological saline solution,
5   dextrose or other saccharide solution, or glycols
such as ethylene glycol, propylene glycol or
polyethylene glycol. When administered in liquid
form, the pharmaceutical composition contains from
about 0.5 to 90% by weight of the synthetic
10 oligonucleotide and preferably from about 1 to 50%
synthetic oligonucleotide.

When a therapeutically effective amount of
synthetic oligonucleotide of the invention is
15 administered by intravenous, cutaneous or
subcutaneous injection, the synthetic
oligonucleotide will be in the form of a pyrogen-
free, parenterally acceptable aqueous solution.
The preparation of such parenterally acceptable
20 solutions, having due regard to pH, isotonicity,
stability, and the like, is within the skill in
the art. A preferred pharmaceutical composition
for intravenous, cutaneous, or subcutaneous
injection should contain, in addition to the
25 synthetic oligonucleotide, an isotonic vehicle
such as Sodium Chloride Injection, Ringer's
Injection, Dextrose Injection, Dextrose and Sodium
Chloride Injection, Lactated Ringer's Injection,
or other vehicle as known in the art. The
30 pharmaceutical composition of the present
invention may also contain stabilizers,
preservatives, buffers, antioxidants, or other
additives known to those of skill in the art.

The amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature

5   of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending

10   physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic effect is obtained for the

15   patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 1.0 ng to about 2.5 mg of

20   synthetic oligonucleotide per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of

25   the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the synthetic oligonucleotide will be in the range of 12 to 24

30   hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

The oligonucleotides of the invention may also be a part of kits for inhibiting HBV replication and infection in a cell. Such a kit includes a synthetic oligonucleotide specific for
5   HBV nucleic acid, such as those described herein. For example, the kit may include at least one of the synthetic contiguous oligonucleotides of the invention, such as, but not limited to, those having SEQ ID NO: 1-31 and 42-48. These
10   oligonucleotides may have modified backbones, such as those described above, and may be RNA/DNA hybrids containing, for example, at least one 2'-O-methyl. The kit of the invention may optionally include buffers, cell or tissue preparation
15   reagents, cell or tissue preparation tools, vials, and the like.

Other kits of the invention are for detecting the presence of HBV in a sample, such as a
20   solution or biological sample, such as a fluid, tissue, tissue homogenate, and the like. These kits contain at least one synthetic oligonucleotide complementary to contiguous or noncontiguous regions of HBV RNA, and means for
25   detecting the oligonucleotide hybridized with the nucleic acid if HBV is present in the sample.

The following examples illustrate the preferred modes of making and practicing the
30   present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1.   Oligonucleotide Synthesis

5   Oligonucleotides were synthesized using
standard phosphoramidite chemistry (Beaucage
(1993) *Meth. Mol. Biol.* **20**:33-61) on either an ABI 394
DNA/RNA synthesizer (Perkin-Elmer, Foster City,
CA), a Pharmacia Gene Assembler Plus (Pharmacia,
10   Uppsala, Sweden) or a Gene Assembler Special
(Pharmacia, Uppsala, Sweden) using the
manufacturers' standard protocols and custom
methods. The custom methods served to increase
the coupling time from 1.5 min to 12 min for the
15   2'-O-methyl RNA amidites. The Pharmacia
synthesizers required additional drying of the
amidites, activating reagent and acetonitrile.
This was achieved by the addition of 3 Å molecular
sieves (EM Science, Gibbstown, NJ) before
20   installation on the machine.

DNA ß-cyanoethyl phosphoramidites were
purchased from Cruachem (Glasgow, Scotland). The
DNA support was 500 Å pore size controlled pore
25   glass (CPG) (PerSeptive Biosystems, Cambridge, MA)
derivatized with the appropriate 3' base with a
loading of between 30 to 40 mmole per gram. 2'-O-
methyl RNA ß-cyanoethyl phosphoramidites and CPG
supports (500 Å) were purchased from Glen Research
30   (Sterling, VA). For synthesis of random
sequences, the DNA phosphoramidites were mixed by
the synthesizer according to the manufacturer's
protocol (Pharmacia, Uppsala, Sweden).

All 2'-O-methyl RNA-containing oligonucleotides were synthesized using ethylthiotetrazole (American International Chemical (AIC), Natick, MA) as the activating
5   agent, dissolved to 0.25 M with low water acetonitrile (Aldrich, Milwaukee, WI). Some of the DNA-only syntheses were done using 0.25 M ethylthiotetrazole, but most were done using 0.5 M 1-H-tetrazole (AIC). The sulfurizing reagent used
10   in all the PS oligonucleotides was 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage Reagent, R.I. Chemical, Orange, CA, or AIC, Natick, MA) as a 2% solution in low water acetonitrile (w/v).

15   After completion of synthesis, the CPG was air dried and transferred to a 2 ml screw-cap microfuge tube. The oligonucleotide was deprotected and cleaved from the CPG with 2 ml ammonium hydroxide (25-30%). The tube was capped
20   and incubated at room temperature for 20 minutes, then incubated at 55°C for 7 hours. After deprotection was completed, the tubes were removed from the heat block and allowed to cool to room temperature. The caps were removed and the tubes
25   were microcentrifuged at 10,000 rpm for 30 minutes to remove most of the ammonium hydroxide. The liquid was then transferred to a new 2 ml screw cap microcentrifuge tube and lyophilized on a Speed Vac concentrator (Savant, Farmingdale, NY).
30   After drying, the residue was dissolved in 400 μl of 0.3 M NaCl and the DNA was precipitated with 1.6 ml of absolute EtOH. The DNA was pelleted by centrifugation at 14,000 rpm for 15 minutes, the supernatant decanted, and the pellet dried. The

DNA was precipitated again from 0.1 M NaCl as described above. The final pellet was dissolved in 500 μl H_2O and centrifuged at 14,000 rpm for 10 minutes to remove any solid material. The

5 supernatant was transferred to another microcentrifuge tube and the amount of DNA was determined spectrophotometrically. The concentration was determined by the optical density at 260 nm. The E_{260} for the DNA portion of

10 the oligonucleotide was calculated by using OLIGSOL (Lautenberger (1991) *Biotechniques* **10**:778-780). The E_{260} of the 2'-O-methyl portion was calculated by using OLIGO 4.0 Primer Extension Software (NBI, Plymouth, MN).

15

Oligonucleotide purity was checked by polyacrylamide gel electrophoresis (PAGE) and UV shadowing. 0.2 OD_{260} units were loaded with 95% formamide/H_2O and Orange G dye onto a 20%

20 denaturing polyacrylamide gel (20 cm x 20 cm). The gel was run until the Orange G dye was within one inch of the bottom of the gel. The band was visualized by shadowing with shortwave UV light on a thin layer chromatography plate (Keiselgel 60

25 F254, EM Separations, Gibbstown, NJ).

Some oligonucleotides were synthesized without removing the 5'-trityl group (trityl-on) to facilitate reverse-phase HPLC purification.

30 Trityl-on oligonucleotides were dissolved in 3 ml water and centrifuged at 6000 rpm for 20 minutes. The supernatant was filtered through a 0.45 micron syringe filter (Gelman Scientific, Ann Arbor, MI) and purified on a 1.5 x 30 cm glass liquid

chromatography column (Spectrum, Houston, TX) packed with C-18 μBondapak chromatography matrix (Waters, Franklin, MA) using a 600E HPLC (Waters, Franklin, MA). The oligonucleotide was eluted at

5   5 ml/min with a 40 minute gradient from 14-32% acetonitrile (Baxter, Burdick and Jackson Division, Muskegon, MI) in 0.1 M ammonium acetate (J.T. Baker, Phillipsburg, NJ), followed by 32% acetonitrile for 12 minutes. Peak detection was

10   done at 260 nm using a Dynamax UV-C absorbance detector (Rainin, Emeryville, CA).

The HPLC purified trityl-on oligonucleotide was evaporated to dryness and the trityl group was

15   removed by incubation in 5 ml 80% acetic acid (EM Science, Gibbstown, NJ) for 15 minutes. After evaporating the acetic acid, the oligonucleotide was dissolved in 3 ml 0.3 M NaCl and ethanol precipitated. The precipitate was isolated by

20   centrifugation and precipitated again with ethanol from 3 ml 0.1 M NaCl. The precipitate was isolated by centrifugation and dried on a Savant Speed Vac (Savant, Farmingdale, NY). Quantitation and PAGE analysis were performed as described

25   above for ethanol precipitated oligonucleotides.

Standard phosphoramidite chemistry was applied in the synthesis of oligonucleotides containing methylphosphonate linkages using two

30   Pharmacia Gene Assembler Special DNA synthesizers. One synthesizer was used for the synthesis of phosphorothioate portions of oligonucleotides using β-cyanoethyl phosphoramidites method discussed above. The other synthesizer was used

for introduction of methylphosphonate portions.
Reagents and synthesis cycles that had been shown
advantageous in methylphosphonate synthesis were
applied (Hogrefe et al., in *Methods in Molecular Biology*,

5   Vol. 20: *Protocols for Oligonucleotides and Analogs* (Agrawal,
ed.) (1993) Humana Press Inc., Totowa, NJ). For
example, 0.1 M methyl phosphonamidites (Glen
Research) were activated by 0.25 M
ethylthiotetrazole; 12 minute coupling time was

10   used; oxidation with iodine (0.1 M) in
tetrahydrofuran/2,6-lutidine/water (74.75/25/0.25)
was applied immediately after the coupling step;
dimethylaminopyridine (DMAP) was used for the
capping procedure to replace standard N-

15   methylimidazole (NMI). The chemicals were
purchased from Aldrich (Milwaukee, WI).

The work up procedure was based on a
published procedure (Hogrefe et al. (1993) *Nucleic*

20   *Acids Res.* **21**:2031-2038). The product was cleaved
from the resin by incubation with 1 ml of
ethanol/acetonitrile/ammonium hydroxide (45/45/10)
for 30 minutes at room temperature.
Ethylenediamine (1.0 ml) was then added to the

25   mixture to deprotect at room temperature for 4.5
hours. The resulting solution and two washes of
the resin with 1 ml 50/50 acetonitrile/0.1 M
triethylammonium bicarbonate (TEAB), pH 8, were
pooled and mixed well. The resulting mixture was

30   cooled on ice and neutralized to pH 7 with 6 N HCl
in 20/80 acetonitrile/water (4-5 ml), then
concentrated to dryness using the Speed Vac
concentrator. The resulting solid residue was
dissolved in 20 ml of water, and the sample

desalted by using a Sep-Pak cartridge. After
passing the aqueous solution through the cartridge
twice at a rate of 2 ml per minute, the cartridge
was washed with 20 ml 0.1 M TEAB and the product
5 eluted with 4 ml 50% acetonitrile in 0.1 M TEAB at
2 ml per minute. The eluate was evaporated to
dryness by Speed Vac. The crude product was
purified by polyacrylamide gel electrophoresis
(PAGE), desalted using a Sep-Pak cartridge. The
10 oligonucleotide was ethanol precipitated from 0.3
M NaCl, then 0.1 M NaCl. The product was
dissolved in 400 μl water and quantified by UV
absorbance at 260 nm.

15 2.   <u>Luciferase Assay Using Stably Transfected</u>
       <u>Cells</u>

       A.   HBV Antisense Target Constructs
20

       All sequences were derived from HBV subtype
ayw (GenBank accession #J02203) as described by
Galibert et al. *(Nature,* (1979) London, **281**:646-
25 650).

       The HBV polymerase-luciferase fusion pGLpol
construct (FIG.10) was prepared by inserting 35
nucleotides spanning the translation start site of
30 HBVayw polymerase gene (nt 2294-2328) between the
cytomegalovirus immediate early gene promoter and
luciferase reporter gene in the plasmid pGLori
(Roche, Nutley, NJ).

35   Three different HBV-luciferase fusion
constructs were generated incorporating the region

around the HBV subtype ayw epsilon region
(FIG.10). The pGLE construct consists of 71
nucleotides representing the epsilon stem loop
region alone (nt 1843-1913) inserted between the
5   cytomegalovirus immediate early gene promoter and
luciferase reporter gene in the plasmid pGLori.
Translation in this construct should initiate at
the HBV core gene initiation site (nt 1903).

10   The constructs pGLE2 and pGLE3 are shown in
FIG. 10. The constructs consist of 130 nucleotides
representing the precore translation start site
and epsilon stem loop region (nt 1813-1943)
inserted between the cytomegalovirus immediate
15   early gene promoter and luciferase reporter gene
in the plasmid pGLori. In these two constructs
the translation start site of the luciferase gene
was removed. In addition, the HBV core gene
translation start site was mutated in pGLE3 (nt
20   1904 T>C). A complementary mutation was
introduced at nt 1854 (A>G) to maintain the base
pairing in the epsilon stem. In pGLE2 translation
can be initiated from the precore or core
translation start site. In pGLE3 translation can
25   only be initiated at the precore translation start
site.

       The plasmid pHBVE+ was generated by
subcloning a *StuI*, *BamHI* fragment from the plasmid
30   pAM6 (ATCC Ac. No. 45020, American Type Culture
Collection, Rockville, MD), representing HBV
subtype adw nt 1701- nt 34 (GenBank accession
V00866) (Ono et al. (1983) *Nucleic Acids Res.*
11:1747-1757), into pBluescript II SK(+)

(Stratagene, La Jolla, CA). This construct was used in RNase H studies.

B.   Generation of Stably Transfected Cell
5   Lines

The HBV subtype ayw-luciferase gene constructs described above were subcloned by polymerase chain reaction from the respective
10 plasmids and the parent plasmid pGLori into the vector pCR-Script (Stratagene, La Jolla, CA), and further subcloned into the vector pcDNA3 (Invitrogen, San Diego, CA). These constructs were stably transfected using Lipofectamine
15 (GIBCO-BRL, Gaithersburg, MD) into HepG2 cells (ATCC Ac. No. HB 8065, American Type Culture Collection, Rockville, MD; US patent 4,393,133). Several Geneticin (GIBCO-BRL, Gaithersburg, MD)- resistant, luciferase-expressing clones were
20 selected at random for each construct.

C.   Antisense Oligonucleotide Assays

Stably transfected HepG2 cells were seeded
25 into 96 well plates. Lipofectin (GIBCO-BRL, Gaithersburg, MD) was diluted to a concentration of 10 μg/ml in Optimem serum-free medium (GIBCO- BRL, Gaithersburg, MD), and 100 μl dispensed into each well of the 96 well plate. Oligonucleotides
30 were diluted to 5 μM or 25 μM in 10 μg/ml Lipofectin in Optimem, and 25 μl dispensed into three wells of the 96 well plate. The oligonucleotide was serially diluted in five fold increments down the plate. The plates were
35 incubated overnight at 37°C. Cells were washed

twice with Dulbecco's phosphate-buffered saline
(PBS) and lysed in 50 μl cell lysis buffer
(Analytical Luminescence Laboratory, San Diego,
CA). Luciferase activity was measured in 20 μl

5   lysate using Analytical Luminescence Laboratory
substrates in a MicroLumat LB 96 P luminometer
(EG&G Berthold, Nashua, NH).

3.   RNase H Cleavage Assay

10

    A.   Preparation of Labelled RNA

    Uniformly ^{32}P-labelled RNA was prepared
from 1 μg linearized plasmid using the Ambion

15   MEGAscript In Vitro Transcription Kit (Ambion,
Inc., Austin, TX) according to the manufacturers'
instructions, using [α-^{32}P]CTP as the radioactive
label. The RNA was treated with RNase-free DNase
I (Ambion, Inc., Austin, TX), extracted with

20   phenol:chloroform: isoamyl alcohol (25:24:1) and
purified from nucleotides and nucleosides on a G-
50 Sephadex spin column (Boehringer-Mannheim,
Indianapolis, IN, or Pharmacia, Uppsala, Sweden).

25   5' end-labelled RNA was prepared from 1 μg
linearized plasmid using the Ambion MEGAscript In
Vitro Transcription Kit (Ambion, Inc., Austin, TX)
according to the manufacturers' instructions,
except that the GTP concentration was lowered to

30   6 mM, and 6 mM guanosine hydrate was added to the
transcription mix. The RNA was treated with
RNase-free DNase I (Ambion, Inc., Austin, TX),
extracted with phenol:chloroform:isoamyl alcohol
(25:24:1) and purified from nucleotides and

nucleosides on a G-50 Sephadex spin column
(Boehringer-Mannheim, Indianapolis, IN, or
Pharmacia, Uppsala, Sweden). The RNA was end-
labelled with [γ-^{32}P]ATP (Amersham, Arlington
5   Heights, IL) and T4 polynucleotide kinase
(Pharmacia, Uppsala, Sweden)according to the
enzyme manufacturers' instructions. The labelled
RNA was purified from nucleosides and nucleotides
on a G-50 Sephadex spin column (Boehringer-
10   Mannheim, Indianapolis, IN, or Pharmacia, Uppsala,
Sweden) and stored at -80°C until needed.

B.   RNase H Cleavage with Random 20mer
Library
15

End-labelled RNA (20-100 nM) was incubated
with a 20 base random DNA library (50-100 μM)
(synthesized on Pharmacia Gene Assembler, as
described above), boiled previously to dissociate
20   any aggregates, for 90 min at 37°C in 9 μl 1 x
buffer (40 mM Tris-HCl pH 7.4, 4 mM MgCl$_2$, 1 mM
DTT). RNase H (Boehringer-Mannheim, Indianapolis,
IN) (1 μl, 1 unit/μl) was then added. The
reaction was incubated at 37°C for 10 min,
25   quenched by addition of 10 μl 90% formamide
containing 0.1% phenol red/0.1% xylene cyanol, and
frozen on dry ice. The quenched reactions were
boiled for 2.5 to 3 minutes, quenched on ice, and
5 to 7 μl loaded onto a denaturing 4%
30   polyacrylamide gel prerun to 50 to 55°C. The
phenol red was typically run to the bottom of the
gel, which was then dried at 80°C under vacuum.
The gel was autoradiographed using XOMAT film
(Kodak, Rochester, NY) or analyzed using
35   phosphorimage technology on a Molecular Dynamics

(Sunnyvale, CA) or Bio Rad Phosphorimager
(Hercules, CA).

    C.   Cleavage of HBV RNA with Semirandom
5   Oligonucleotides

    Semirandom oligonucleotides (100 μM in H_2O)
were boiled for 1 min to dissociate any aggregates
formed between complementary sequences in the mix
10 and 1 μl (final concentration 10 μM) was added to
8 μl 1 x RNase H buffer (40 mM Tris-HCl pH 7.4, 4
mM $MgCl_2$, 1 mM DTT) containing end-labelled RNA
(20-100 nM). After a 15 minute preincubation at
37°C, RNase H was added (1 U) and incubated for 10
15 min at 37°C. The reactions were quenched and
analyzed as described above. Sites of cleavage
were estimated using DNA and/or RNA molecular size
markers.

20   D.   Cleavage of HBV RNA with Specific
              Antisense Oligonucleotides

    In 9 μl 1 x RNase H buffer (40 mM Tris-HCl pH
7.4, 4 mM $MgCl_2$, 1 mM DTT), 20-100 nM labelled RNA
25 and 100 nM oligonucleotides were preincubated for
15 min at 37°C. 1 μl RNase H (1 U/μl) was added,
and the reaction was incubated at 37°C for 10 min.
The reactions were quenched and analyzed as
described above.
30

    Quantitation of the cleavage products was
performed using software supplied with the
Phosphorimager (Molecular Dynamics, Sunnyvale, CA,
or Bio-Rad Laboratories, Hercules, CA). "Counts"
35 were determined by drawing a box around the band
of interest and subtracting the background

determined with a box drawn nearby. Counts in a product band were compared to total counts in the lane above that band to determine % cleavage.

5 4. HBV Encapsidation Assay

The assay is essentially identical to that described in Pollack et al. (*J. Virol.* (1993) **67**:3254-3263). Briefly, HepG2 cells are

10 transfected with the plasmids pCMV-CP and pE-LacZ (Dr. D. Ganem, University of California Medical Center, San Francisco, CA) by calcium phosphate precipitation. The HepG2 cells are treated with 0-10 μM antisense oligonucleotides pre- or post-

15 transfection. Three days after transfection the cells are harvested and total cell RNA is prepared using Trizol reagent (GIBCO-BRL, Gaithersburg, MD). Alternatively, HBV core particles are collected from cytoplasmic extracts after nuclease

20 digestion by polyethylene glycol precipitation. The encapsidated RNA is extracted from the core particles using Trizol reagent (GIBCO-BRL, Gaithersburg, MD).

25 The relative amounts of E-LacZ RNA in total cell RNA and encapsidated in core particles are assessed using a ribonuclease protection assay (RPA) (Ambion, Austin, TX) using RNase T1. The RNA probe used is transcribed by T7 polymerase

30 (Ambion, Austin, TX) from the plasmid pLacProbe. The plasmid pLacProbe was constructed by subcloning a 425 bp *Mlu* I fragment from pE-LacZ into the vector pGEM3z (Promega, Madison, WI).

Data from the RPA is quantitated using a BioRad
GS250 Phosphorimager (BioRad, Hercules, CA).

5.   Studies of Oligonucleotide Anti-Viral
     Activity by Southern Hybridization Analysis

     A.   Cell Culture

     The cell line HepG2.2.15 (Sells et al. (1988)
J. Virol. 62: 2836-2844) was routinely cultured in
RPMI.1640 medium (Life Technologies Ltd., Paisley,
Scotland) supplemented with 10% fetal bovine serum
(FBS), 100 U/ml penicillin, and 150 μg/ml
streptomycin. Cultures were replaced after 10
passages with cells freshly cultured from a
mycoplasma-free frozen stock. Cultures were
maintained at 37°C in a humidified atmosphere of
5% CO_2 in air.

     Cells were cultured in 6-well plates at 10^6
cells/well in 4 ml medium (RPMI.1640 as above but
supplemented with 5% FBS) and maintained as above.
After 2 days, the medium was replaced with fresh
medium containing 3% FBS and antiviral compound.
For antisense experiments, cells were treated with
a series of five 3-fold dilutions from a starting
concentration of 10 μM. Cultures with 3TC were
treated in a similar fashion but with an initial
concentration of 1.0 μM. The cultures were
maintained for 10 days, during which medium and
compound was replaced after 3, 5 and 7 days.
Cells were washed once with Hanks balanced salt
solution (HBSS) immediately prior to each
replacement. After 10 days, the cells were washed
twice with HBSS and treated overnight at 37°C with

0.55 ml lysis buffer (10 mM Tris. HCl pH 7.5; 5 mM
EDTA; 150 mM NaCl; 1.0% w/v sodium dodecyl
sulphate) containing 100 μg/ml proteinase K. The
lysate was harvested, treated for 1 hour at 60°C,

5   and extracted once with phenol/chloroform and
twice with chloroform before precipitation twice
with ethanol. The dried precipitate was
resuspended in 50 μl TE buffer (10 mM Tris-HCl, pH
8.0; 1 mM EDTA) and allowed to dissolve overnight

10   at 4°C. The solution was then assayed for DNA by
spectrophotometric measurement at 260 nm. Yields
were of the order of 40 to 100 μg with 260/280
ratios within the range 1.5 to 2.0.

15   B.   Electrophoresis and Blotting

DNA obtained from HepG2.2 15 culture was
digested in 20 μg amounts in 30 μl buffer E with
10 units of HindIII (Promega Limited, Southampton,

20   U.K.) overnight at 37°C. The DNA fragments were
separated by agarose gel electrophoresis on 0.8%
gels run in 0.5 x TBE at 50 volts overnight. Gels
were then treated serially with 0.25 M HCl for 20
minutes; 0.5 M NaOH in 1.0 M NaCl for 45 minutes,

25   and finally with 0.5 M Tris-HCl, pH 7.0, and 1.0 M
NaCl for 30 minutes, all at room temperature with
gentle shaking. The gels were rinsed with 6 x SSC
and the DNA blotted overnight onto nylon membranes
(Hybond N; Amersham International, Bucks, U.K.) by

30   capillary action. The membranes were washed with
6 x SSC for 5 minutes and dried before UV cross-
linking using a Stratalinker (StrataGene Limited,
Cambridge, U.K.). The blots were stored at 4°C
until hybridized.

C.   Preparation of Southern Hybridization
      Probe

5   A full length HBV genome fragment was
      prepared from the plasmid pCH3/3097
      (Bartenschlager et al. (1992) Nucleic Acids Res.
      20:195-202) by means of excision with restriction
      endonucleases HindIII, SacI and PvuI in buffer C
      (Promega Limited, Southampton, U.K.), followed by
10   agarose gel electrophoresis purification. This
      fragment was used to produce the labelled probe by
      random-primed DNA synthesis in the presence of
      ^{32}P-dCTP (Amersham AA0005 [α-^{32}P]dCTP 110 TBq/mmol
      (3000 Ci/mmol), in stabilized aqueous solution
15   with dye) using the "Megaprime" kit (Amersham
      International, Bucks, U.K.). A starting amount of
      25 ng HBV DNA was labelled to an estimated
      specific activity of 1-2 x 10^9 dpm/μg DNA.

20   D.   Hybridization

      Membranes were pre-hybridized with formamide
      solution supplemented with 100 μg/ml heat-
      denatured salmon-sperm DNA(Sigma-Aldrich, Poole,
25   U.K.) in a hybridization oven at 42°C for at least
      3 hours. The solution was replaced with fresh
      formamide solution (10.0 ml) supplemented with
      salmon-sperm DNA as before and freshly prepared
      ^{32}P-labelled probe. Incubation was continued for
30   a further 16-20 hours. The probe was removed and
      the membranes washed with 2 x SSC supplemented
      with 0.1 % (w/v) SDS twice for 15-30 minutes at
      65°C. Washes were repeated with 1 x SSC and 0.5 x
      SSC all supplemented with 0.1% SDS. The blots
35   were examined for background label using a Mini

Monitor (MiniInstruments Ltd., Burnham-on-Crouch, U.K.) and, if further washes were not required, the membranes were dried, wrapped with plastic film (Saran Wrap), and placed in a cassette for phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

E.   Analysis

After 1-3 days exposure, results were obtained using a Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA). Analysis was carried out using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). Those bands representing integrated DNA (10 kb) and completed replicative intermediate (RI - 3.8 kb), as described by Sells et al. (*J. Virol.* (1988) **84**:1005-1009), were identified by reference to a marker lane containing a 1 kb DNA ladder. The amount of replicative intermediate present relative to integrated DNA was calculated (3.8 kb DNA/10.2 kb DNA) and percent inhibition calculated according to the formula:

$$\% \text{ inhibition} = 100 - \left\{ \left(\frac{relative\ amount\ of\ RI\ in\ treated\ culture}{relative\ amount\ of\ RI\ in\ untreated\ culture} \right) x100 \right\}$$

The concentration of compound which produced 50% inhibition of RI formation (IC_{50}) was determined graphically.

6.   Kinetic PCR Protocol for HBV Anti-Viral Assay

     The anti-viral assay was performed using HepG
2.2.15 cells (Sells et al. (1986) *Proc. Natl. Acad. Sci.*
5 *(USA)* **84**:1005-1009) seeded at a density of 1 x 10^5
per well in 24 well plates. The cells were grown
to confluence and allowed to stabilize for 2-3
days in RPMI media (supplemented with 10% fetal
calf serum (FCS), 2 mM glutamine and penstrep
10 (Life Technologies Ltd., Paisley, Scotland) prior
to the addition of the oligonucleotide (defined as
day 0). Six dilutions (10, 5, 1, 0.5, 0.1, and 0
μM) were set up in duplicate for each of the
antisense oligonucleotides. In each assay run,
15 the nucleoside analog, β-L-(2R,5S)-1,3
oxathiolanyl cytosine (3TC) (Glaxo, Greenford,
U.K.) was included as positive control at 0.5,
0.1, 0.075, 0.05, and 0.01 and 0 μM in duplicate.
Oligonucleotide was added to 1 ml of RPMI media
20 (supplemented with 3% FCS, 2 mM glutamine and
penstrep) at each of the indicated dilutions. At
days 2, 4 and 7, the old media was removed and
replaced with fresh media containing compound. At
day 10, the supernatants were harvested, clarified
25 by low speed centrifugation, prior to the addition
of Triton X100 (Sigma, St. Louis, MO) and tri-n-
butyl phosphate to give a final concentration of
1%. The samples were then heated to 70°C for 20
minutes to disrupt the viral particles.
30

     Following this treatment, the viral particles
were subject to analysis by kinetic PCR. The
primers RJ407 (SEQ ID NO:51) and RJ431 (SEQ ID

NO:52) were used to detect a 205 bp fragment of
the core gene. Kinetic PCR was performed
essentially as described by Higuchi et al.
(*Biotechnol.* (1993) **11**:1026-1030). Briefly, the PCR
5   reactions were set up under standard conditions
except that ethidium bromide was included at a
concentration of 4 μg/ml. After each PCR cycle,
the samples were illuminated with UV light at 302
nm, and a picture was taken using a computer
10   controlled, cooled CCD video camera with the lens
focused on the surface of the thermocycler block.
A kinetic PCR analysis was performed by plotting
the average intensity of fluorescence from each
PCR sample after each annealing/extension cycle
15   against the cycle number. The original template
concentration can be calculated by utilizing a
standard fluorescence curve generated by templates
of known concentration.

20   7.   *In vitro* Translation Assays

     A.   Construction of pHBVpol

     The 5' end of the polymerase gene (*pol*) open
25   reading frame, nt 2292-2942, was amplified by the
PCR from the full length HBV clone pCH3/3097
(Bartenschlager et al. (1992) Nucleic Acids Res.
20:195-202). The 5' amplification primer AS10
(SEQ ID NO:55) encoded an EcoRI site. The 3'
30   amplification primer AS11 (SEQ ID NO:56) both
encoded a PstI site and introduced a stop codon in
the place of a leucine codon at position 2942-
2944. The PCR product was digested with EcoRI and

PstI (Promega, Madison, WI) and inserted into
similarly digested plasmid vector pGEM-3z
(Promega, Madison, WI). The resulting recombinant
plasmid was recut with PstI and an oligonucleotide
5   dA:dT$_{(30)}$ linker was introduced. A sketch map of
pHBVpol is shown in FIG. 13.

B.   *In vitro* Transcription of RNA

10   pHBVpol and a second plasmid pHSVProt were
linearized with HindIII (Promega, Madison, WI),
RNA was *in vitro* transcribed from each construct
using T7 Cap-Scribe reagents (Boehringer-Mannheim,
Indianapolis, IN) employed as per the
15   manufacturer's instructions. The quantity and
quality of the RNA's was assessed on a 2%
agarose/formaldehyde gel. The control HSVProt RNA
was arbitrarily diluted 10 fold to 200 ng/μl and
stored in 20 μl aliquots at -80°C.
20

C.   Assessment of Antisense Activity of Pol
       Oligonucleotides

The sequences of all oligonucleotides used in
25   these experiments are shown in Table 1. In this
series of experiments only phosphodiester (PO)
oligonucleotides were used. 5 μM, 2.5 μM, and
1.25 μM stocks of each of HBVpol-1, HBVpol-2,
HBVpol-3, and randomer were made up and stored
30   frozen. Reactions were set up using 100 ng HBVpol
and 200 ng pHSVprot. The volume of water in the
translation master mix was reduced to allow the
addition of 1 μl of each dilution of each
oligonucleotide to the reactions whilst
35   maintaining the final volume at 12 μl. This

corresponds to final oligonucleotide
concentrations of approximately 400 nM, 200 nM,
and 100 nM.

5   EQUIVALENTS

        Those skilled in the art will recognize, or
be able to ascertain, using no more than routine
experimentation, numerous equivalents to the
10   specific substances and procedures described
herein. Such equivalents are considered to be
within the scope of this invention, and are
covered by the following claims.